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FURTHER STUDY OF THE ROLE OF CALCIUM IN SYNAPTIC TRANSMISSION

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SUMMARY

1. The effect of calcium on synaptic transmission has been studied by intracellular recording of pre- and post-synaptic potential changes in the stellate ganglion of the squid.

2. For a given presynaptic 'input' (propagated spike, or local depolarizing pulse after tetrodotoxin treatment), the post-synaptic response increases with external calcium concentration $[Ca]_o$ in a highly non-linear fashion, indicating that transmitter output varies with more than the second power of $[Ca]_o$ over a certain concentration range.

INTRODUCTION

The importance of calcium in the release of neural transmitters as well as in many non-neural secretory processes has been well established. There is, however, relatively little information on the quantitative relationship between calcium concentration and amount of transmitter released by the nerve impulse. The question has been studied quantitatively only at the neuromuscular junction, and it is clearly of interest to examine it at a synapse where it is possible to measure pre- as well as post-synaptic potential changes. The present experiments were made on the giant synapse of the squid (*Loligo vulgaris*) extending previous work on this preparation.

METHODS

The procedures of dissecting and mounting the preparation and of inserting microelectrodes into the pre- and post-synaptic elements of the giant synapse in the stellate ganglion of the squid have been fully described elsewhere (Miledi, 1967; Katz & Miledi, 1967). The advantages of this preparation for a study of synaptic transfer are obvious, but there are certain drawbacks when one requires to determine the quantitative effects of different compositions of the bathing medium. The difficulty arises from the slow diffusion equilibration in the stellate ganglion which

usually made it impossible to examine more than one or two calcium concentration changes, with reversal control, in any one preparation.

Two kind of experiments were made. In the first section, post-synaptic potentials were recorded, produced by propagated nerve impulses in either the 'main' pre-axon (which forms the giant synapses), or in an 'accessory' pre-axon (which forms 'proximal' synapses, see Young, 1939). In the second section, impulses were eliminated with tetrodotoxin (TTX), and the 'synaptic transfer' characteristic (input/output curve) was determined in different calcium concentrations, by applying various intensities of brief depolarizing pulses to the presynaptic element of the giant synapse. Ideally, the input/output curve would give us an indirect, but accurate, picture of the relation between amplitude of presynaptic depolarization and amount of transmitter released by the terminal. The observed curve, however, suffers from several non-linear distortions (Katz & Miledi, 1967). One of these arises from delayed rectification (rise of potassium conductance) in the presynaptic membrane which causes the potential to form an initial transient instead of being maintained throughout the current pulse, and the duration of the transient to become briefer as the intensity is increased. This distorting factor has been reduced in earlier experiments by intracellular application of tetraethylammonium (TEA; see Katz & Miledi, 1967). In the present work, however, TEA injection was avoided: the advantage of reducing potassium conductance is outweighed by the development of a regenerative local response (Katz & Miledi, 1969*a*) and also by the risks of progressive development or gradual fading of the TEA action during the course of the experiment. This would introduce an extra variable and make it more difficult to evaluate the quantitative effects of successive changes of calcium concentration.

To minimize any errors due to spatial attenuation of input potentials between the site of recording and sites of transmitter release, the recording pre-electrode was placed into the terminal at the start of the synapse or 0·1 to 0·2 mm further down within the synaptic region itself. The current electrode was inserted about 0·5 mm 'upstream' into the pre-axon, and the post-synaptic recording electrode was placed within the distal portion of the synaptic region.

The experiments were made at low temperature, usually around 11° C. Calcium concentrations were varied between 1 and 22 mM, 11 mM being the normal level. The changes were made by adding various volumes of an isotonic stock solution of $CaCl_2$ (0·39 M) to a calcium-free artificial sea water of the following composition: NaCl 0·58 M, 804 parts; KCl 0·58 M, 18 parts; $MgCl_2$ 0·37 M, 146 parts; $NaHCO_3$ 0·58 M, 4·6 parts.

As in similar previous experiments (Katz & Miledi, 1969*b*), two alternative procedures were used for changing the bath solution: (*a*) when only a single intracellular electrode was used (for recording from the post-axon, as in Fig. 1), this electrode was temporarily withdrawn while the chamber was quickly emptied and flushed twice with the new solution before continuing the slow superfusion; (*b*) when microelectrodes had been inserted in the presynaptic terminal, the fluid was replaced more slowly, to avoid dislodging any electrodes, by changing the continuously running bath perfusion to the new solution.

RESULTS

A. Synaptic response to nerve impulses

Fig. 1 illustrates an experiment in which the calcium concentration was varied between 2 and 11 mM. The results have been plotted on semi-logarithmic co-ordinates; they show a more than 100-fold drop in the

amplitude of the post-synaptic potential when the calcium concentration was lowered from 11 to 2 mM, and at a later stage an approximately twentyfold increase when the concentration was doubled, from 5·5 to 11 mM.

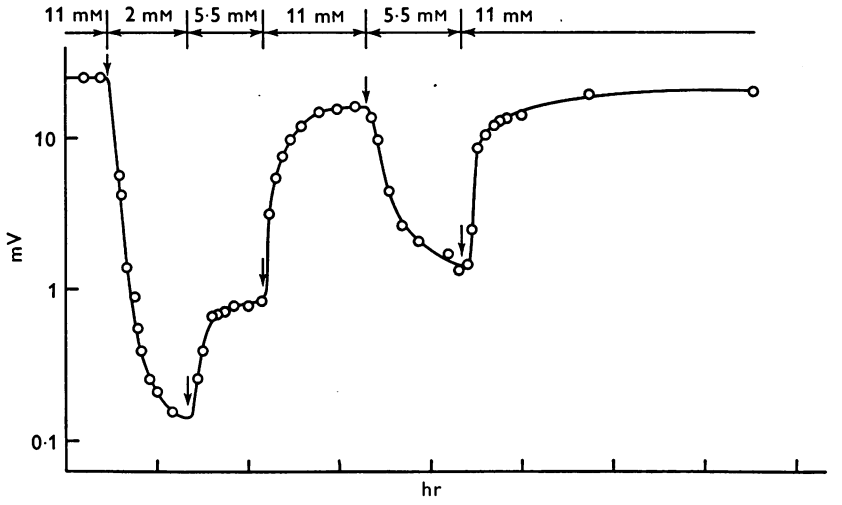

Fig. 1. Effect of external calcium concentration $[Ca]_o$, on post-synaptic potential. At the times indicated, by arrows and vertical bars above the curve, changes of $[Ca]_o$ were made in the following sequence: from 11 mM to 2, 5·5, 11, 5·5, and back to 11 mM.

There are several reasons for assuming that this large change in the post-synaptic potential is mainly, or entirely, due to a change in the amount of transmitter released by the presynaptic nerve impulse. One argument is the close analogy to the effect of calcium at vertebrate myoneural junctions. Here, the post-synaptic sensitivity to the transmitter is only slightly affected, while the quantal output of acetylcholine varies strongly with changes of calcium concentration in a corresponding range (Takeuchi, 1963; Dodge & Rahamimoff, 1967). At the squid synapse, the transmitter has not yet been identified, and direct evidence on post-synaptic sensitivity is therefore not available. There is, however, indirect evidence for its relative constancy under the present experimental conditions, from measurements of miniature synaptic potentials (see Miledi, 1967). These were recorded, during TTX paralysis, from intraganglionic branches of the post-synaptic axons as well as from cell bodies of small motor axons (Miledi, 1967). The amplitudes of the spontaneous potentials were found not to be noticeably changed when the calcium concentration in the bath was altered from 1 to 11 mM. For example, in one post-axon branch the average amplitude of the recorded spontaneous potentials remained at approximately 0·12 mV

in both low and high Ca solutions. When potentials of less than 100 μV amplitude were discarded, the mean value was 182 μV in the low (1 mM) and 195 μV in the high (11 mM) calcium concentration. The largest single potential was 0·4 mV in the low, and 0·54 mV in the high calcium solution. To summarize, it is clear that any effect of calcium on the size of the spontaneous synaptic 'unit' potentials is quite negligible compared with the very large effect on the impulse-evoked synaptic potential. The relative constancy in the size of the miniature potentials indicates that the post-synaptic sensitivity has undergone little or no change.

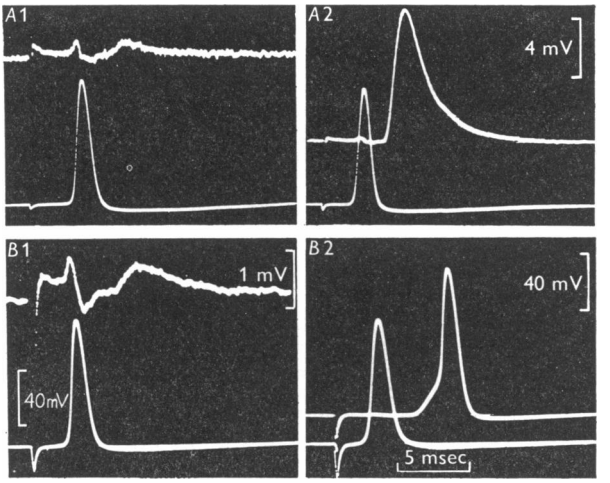

Fig. 2. Simultaneous recording of presynaptic spike (lower trace of each block) and post-synaptic potential (upper trace). *A* and *B*, from two different experiments; *A*: recording from 'main' pre-axon, about 0·5 mm from start of giant synapse; *B*: recording from 'accessory' pre-axon. *A* 1 in 1 mM-Ca, *A* 2 after raising $[Ca]_o$ to 4 mM. *B* 1 in 2·75 mM-Ca, *B* 2 after raising $[Ca]_o$ to 22 mM. Calibrations: 40 mV scale in *B* 1 applies to all presynaptic recordings. 1 mV scale applies to post-synaptic potentials in *A* 1 and *B* 1. Post-synaptic recording in *A* 2 and *B* 2 was made at the lower amplifications shown by the corresponding (4 and 40 mV) scales.

Miledi & Slater (1966) have shown (by further analogy to the neuromuscular junction) that blockage of synaptic transmission during removal of external calcium occurs without failure of nerve conduction in preterminal or terminal axon branches. Figs. 2 and 3 confirm these findings and show, by simultaneous intracellular recording from pre- and post-synaptic elements, that the calcium effect cannot be ascribed to a change in the size of the presynaptic spike. Transient variation or gradual drift of the amplitude and duration of the pre-spike did occur (in some cases caused by temperature variation during solution changes). But these were

erratic effects which were not correlated with the consistent action of calcium on the post-synaptic response. The large increase of the latter, during return from low to high $[Ca]_o$, was observed even when there was a concomitant decline of the pre-spike amplitude by several millivolts.

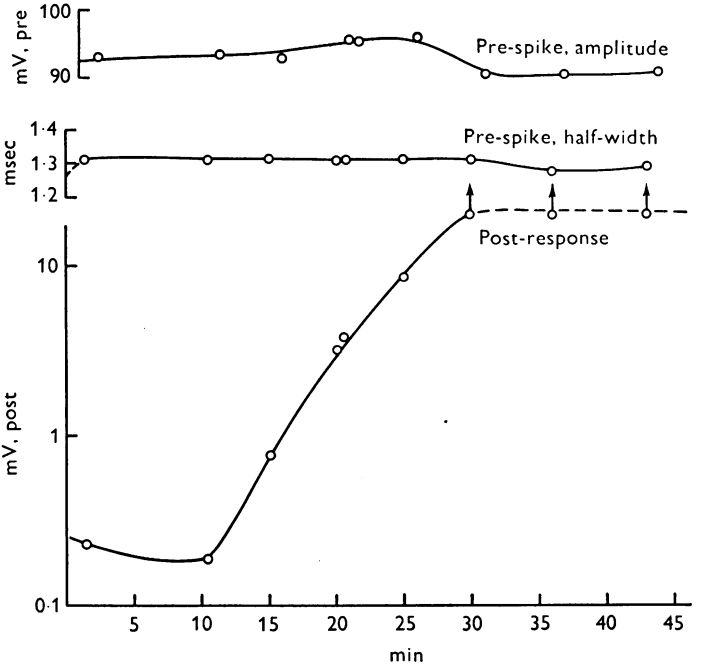

Fig. 3. Presynaptic spike amplitude (top curve) and spike duration at 50 % peak height (middle curve), and post-synaptic potential (lower curve), after raising $[Ca]_o$ in the perfusion fluid at time zero from 2·75 to 22 mM. Same experiment as in Fig. 2*B*. Pre-spike values are plotted on linear ordinate scales, post-potential on logarithmic scale. Arrows indicate superthreshold amplitude of post-potential. Note: 100-fold increase of post-potential as a result of rise in $[Ca]_o$, without change in pre-spike.

B. The 'input/output' relation in different calcium concentrations

After treatment with TTX, depolarizing pulses were applied directly to the presynaptic terminals to determine the 'synaptic transfer' curve (cf. Katz & Miledi, 1967). After each change of $[Ca]_o$ in the perfusing fluid, input/output series were recorded periodically, at intervals of 10–20 min, until the effect of the change had become more or less stable. An experiment of this kind is illustrated in Fig. 4, showing the large increase in the post-synaptic response to a given presynaptic potential change, when the calcium concentration in the bath was raised from 2·75 to 22 mM. Results from this experiment are plotted, on a linear scale, in Fig. 5. Results from

another experiment are shown, on semilogarithmic co-ordinates, in Fig. 6. With regard to the latter, it must be pointed out that amplitudes smaller than 0·5 mV could not be measured very accurately and the initial slopes, therefore, especially in the low calcium solution, remain somewhat un-

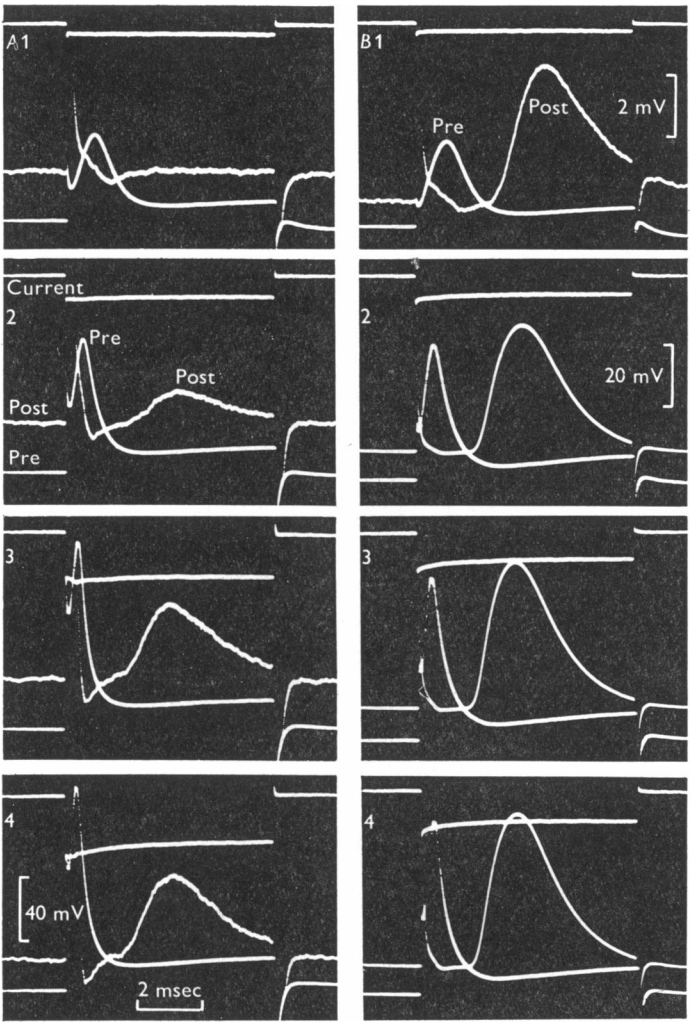

Fig. 4. Effect of $[Ca]_o$ on synaptic transfer. *A*: in 2·75 mM-Ca; *B*: in 22 mM-Ca. 1–4: with increasing depolarizing input pulses. In each block, top trace shows current pulse, middle trace post-synaptic potential, bottom trace presynaptic potential. Calibrations: The 2 mV scale applies to the post-potentials in *A* 1–*A* 4 and *B* 1; the 20 mV scale to post-potentials in *B* 2–*B* 4. The 40 mV scale applies to all pre-potentials. The scale in *B* 1 also represents 3·7 μA for the current calibration.

certain. It is clear, however, that over a wide range of input potentials, the transmitter output (measured by the post-synaptic response) varies much more than in linear proportion to the calcium concentration.

As the input potentials are increased to 100 mV and more, the post-synaptic response approaches a ceiling at high $[Ca]_o$, while still rising at

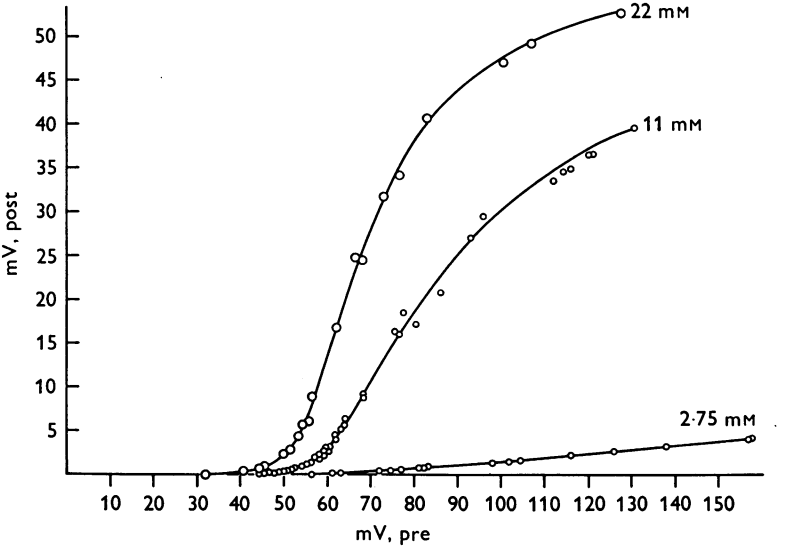

Fig. 5. Input/output curves in different calcium concentrations. The three curves were obtained with 22, 11 and 2·75 mM-Ca, starting with the middle curve (11 mM) and finishing with the top curve (22 mM).

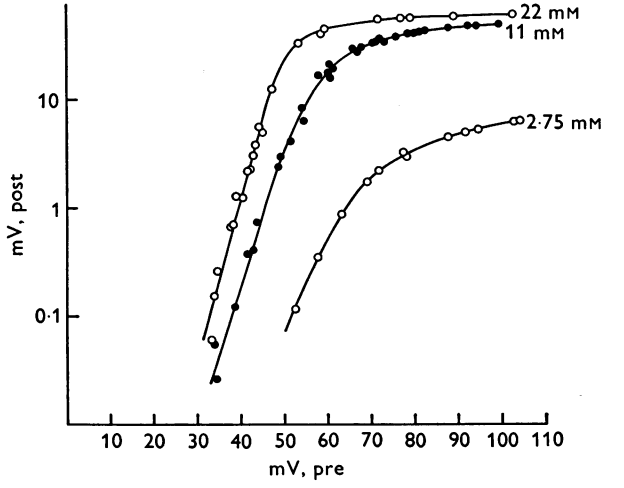

Fig. 6. Input/output curves, from a similar experiment, on semilogarithmic co-ordinates, using 22, 11 and 2·75 mM-Ca.

low $[Ca]_o$ (Figs. 5 and 6). The 'ceiling' can be explained partly by the limitation imposed on the growth of the response post-synaptically, as it approaches the 'equilibrium potential' (see Miledi, 1969). An approximate correction can be made for this non-linear distortion, as shown, for example, in Fig. 8 below. It is apparent, however, even from the uncorrected input/output curves, that the very large reduction in the maximum output, brought about by lowering $[Ca]_o$, cannot be compensated by increasing the input voltage.

It has previously been shown (Bloedel, Gage, Llinás & Quastel, 1967; Katz & Miledi, 1967) that a 'conditioning' hyperpolarization (H) of the pre-axon can facilitate transmitter output evoked by a subsequent depolarizing pulse, an effect which resembles somewhat that of raising the calcium concentration (Katz & Miledi, 1967, p. 425). In one of the present experiments (that illustrated in Fig. 4) conditioning H pulses were applied in order to test whether the reduced response in low $[Ca]_o$ (2·75 mM) could be restored in this way to the normal level. The H pulses varied in duration between 10 and 500 msec, and in amplitude between 20 and nearly 60 mV. The effect was to raise the output for a given presynaptic *net* depolarization (measuring the latter always from the resting potential) by a factor 2 to 3, depending on the amplitude of the H pulses. This effect is similar in magnitude to that described previously at normal calcium concentration (Katz & Miledi, 1967, Fig. 14). It was clearly not possible to recoup in this way more than a small fraction of the response obtainable in the normal calcium solution. Moreover, part of this relatively small restoration must be attributed, not to the hyperpolarization directly, but to a lengthening in duration of the input potential which followed the H pulse (see Katz & Miledi, 1967, pp. 423 and 425).

The question may be raised whether small changes in presynaptic membrane resistance and resting potential were contributory factors in producing the large calcium effect. In low $[Ca]_o$, the resting potential tends to fall, but in the present experiments the decline amounted at most to a few millivolts and, in fact, did not exceed the error of measurement. The effect of such small changes in resting potential can probably be ignored, considering that much larger hyperpolarizations have only a weak restoring action.

An estimate of changes in membrane resistance was obtained by plotting the presynaptic voltage/current (V/I) relation. In the experiment of Fig. 4, the V/I ratio fell in low $[Ca]_o$, but the decline amounted to only about 3% even for the largest input voltages. In the experiment of Fig. 6, the V/I ratio fell by about 5·5% on reducing $[Ca]_o$ from 11 to 2·75 mM, but declined by a further 3% on raising $[Ca]_o$ from 2·75 to 22 mM; yet the recovery of the response during the rise of $[Ca]_o$ was equally striking in both

experiments (cf. Figs. 5–7). One may conclude, therefore, that the changes in resting potential and membrane resistance were too small to make an important contribution to the $[Ca]_o$ effects observed in these experiments.

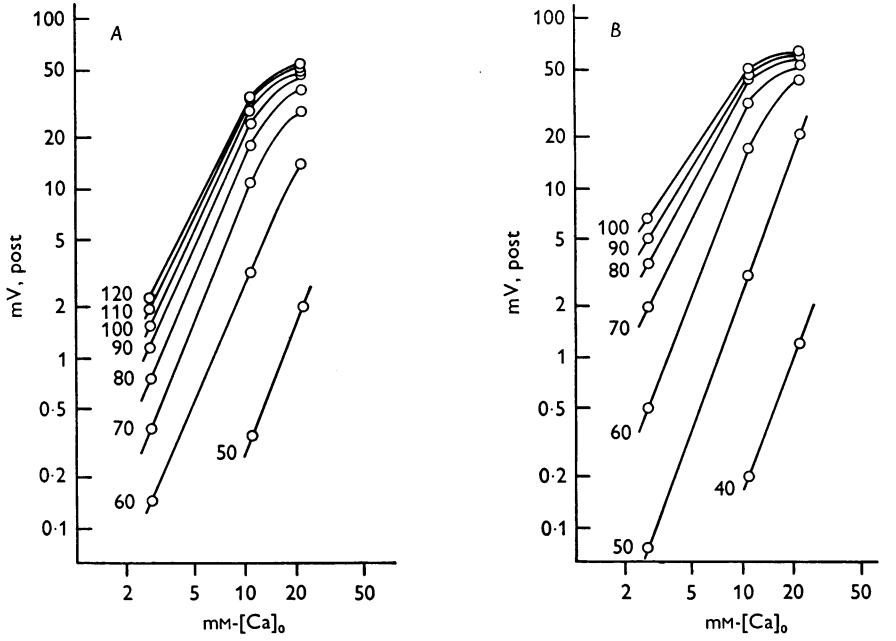

Fig. 7. Relation between $[Ca]_o$ and post-synaptic response, at various presynaptic input potentials (which are shown, in mV, by the number against each curve). The data were taken from the experiments of Figs. 5 and 6, by reading off the family of semilogarithmic curves (cf. Fig. 6) at the indicated values of the pre-potentials. Maximum slopes of the log response/log $[Ca]_o$ curves are approximately 2·5.

In Fig. 7, results of the two experiments represented in Figs. 5 and 6 have been re-plotted on double-log scales to show the relation between calcium concentration and post-synaptic response to a given presynaptic input. In the range of low amplitudes (with post-synaptic responses of less than 10–20 mV), the slopes attain nearly constant values in both experiments, of 2·4 and 2·6 respectively. This would suggest that over a certain range, transmitter output increased with approximately the 2·5th power of the calcium concentration. From the examples quoted in Section *A*, it would appear that this power index can vary considerably in different experiments, and that transmitter release following a nerve impulse may increase with as much as the 4th or even higher power of $[Ca]_o$.

DISCUSSION

In most of the earlier work on the relation between $[Ca]_o$ and transmitter release, the conclusions rested on post-synaptic recording alone, and the assumption had to be made that the presynaptic input (e.g. nerve action potentials, or potassium-induced depolarization) did not vary with changes in $[Ca]_o$. One of the objects of the present study was to check this point by recording directly from both sides of the synapse. The results confirm that calcium is needed to evoke transmitter release by depolarization of the presynaptic terminal. Furthermore, the relation between response and external calcium concentration is very steep and resembles the power function found at the vertebrate neuromuscular junction (Jenkinson, 1957; Dodge & Rahamimoff, 1967; Hubbard, Jones & Landau, 1968).

Before discussing the results in detail, it is necessary to consider residual sources of error which might affect the interpretation. The input/output curves suffer from a number of inaccuracies and give only a distorted picture of the relationship between presynaptic depolarization and transmitter release (see Katz & Miledi, 1967). This is (*a*) because the post-synaptic potential is not proportional to transmitter output except over a small range (see Martin, 1955), and (*b*) because the duration of pre-potentials is dependent on their amplitude and diminishes as the latter increases (see Fig. 4). Transmitter release is known to be affected by both variables (Katz & Miledi, 1967); the input/output curves, therefore, are clearly in need of correction, for they show the response as a function of pre-potential amplitude alone. The effect of such a correction can be stated only in qualitative terms: it will make the output potential rise rather more steeply than in the uncorrected curves.

The situation becomes much simpler if we confine ourselves to an assessment of the calcium effect on transmitter release *at constant input potential*, as represented by the family of curves in Fig. 7. If one further restricts consideration to the lower range of responses, in which post-synaptic distortion can be ignored, the uncorrected data may be accepted directly, as at least an approximate index of the relation between $[Ca]_o$ and transmitter release.

To cover a wider range of responses the effect of post-synaptic distortion (i.e. factor (*a*) above) may be tentatively corrected for, by multiplying the recorded post-synaptic potential v by the factor $V/(V-v)$, where V is the theoretical upper limit of v (Martin, 1955). With a resting potential of -65 mV, and a post-synaptic equilibrium potential of $+20$ mV (Miledi, 1969), $V = 85$ mV. This correction factor has been applied to the data of Fig. 7, with the expected result (Fig. 8) that the uppermost curves become straighter and steeper, and show parallel alignment to the lower

ones. The maximum slopes are only slightly increased, to 2·5 and 2·7 respectively.

While the consistent relationship in these two experiments is of interest, no very great accuracy can be claimed for these results. Minor errors may have been introduced by small changes, with varying $[Ca]_o$ in presynaptic

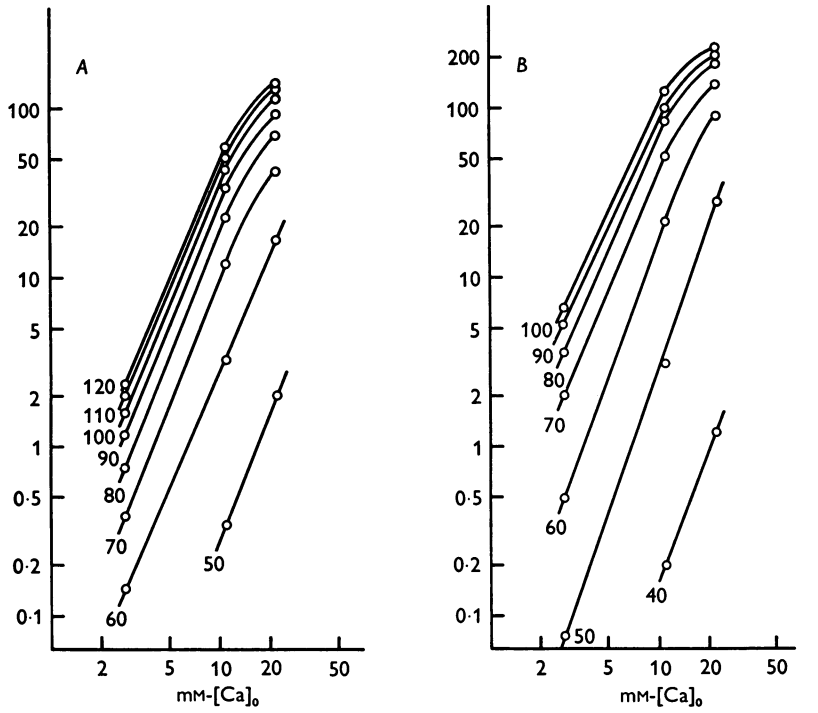

Fig. 8. Fig. 7 has been replotted, using the 'correction factor' $V/(V-v)$ for the ordinate values, where v is observed amplitude of post-synaptic potential and V its upper limit (taken as 85 mV).

resting potential and membrane resistance (the latter introducing small differences in duration of input potentials of equal amplitude). Furthermore, equilibration in the different calcium concentrations was a slow process; and while it was checked by repeated tests extending over 1½–2 hr perfusion periods in each solution, some uncertainty about full completion of equilibration remains. Finally, the sequence of observations, namely 11 decreasing to 2·75 and then rising to 22 mM-$[Ca]_o$, did not entirely eliminate the possibility of inaccuracies due to progressive drift in synaptic properties.

In spite of these reservations, the results are sufficiently suggestive to merit discussing some theoretical implications. On the current calcium hypothesis, transmitter release is brought about by influx of external calcium ions through special membrane channels which are 'opened' by

the depolarizing pulse. Suppose the influx of calcium is given by $M_{Ca} = k[Ca]_o$, where k is a voltage- and time-dependent permeability coefficient. Suppose further, as suggested by Dodge & Rahamimoff (1967), that a release site is activated by simultaneous action of n calcium ions. In that case, the relation between transmitter release R and M will follow nth power Michaelis–Menten kinetics, and its initial slope, for small values of M, will be given by $d(\log R)/d(\log M) = n$. For a given input voltage $d(\log R)/d(\log [Ca]_o) = n$, while at constant $[Ca]_o$ and varying input voltage, $d(\log R)/d(\log k) = n$.

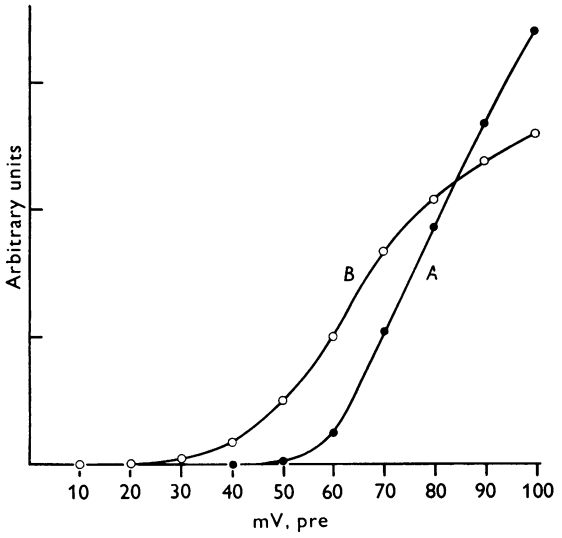

Fig. 9. Relations between presynaptic depolarization, and (*A*) transmitter release R (filled circles), (*B*) 'Calcium permeability coefficient' k (open circles). The data for *A* have been taken from the results of Fig. 8*B*, at 2·75 mM-$[Ca]_o$. Curve *B* has been derived as described in the text, assuming an exponential rise of the curve up to 50 mV. Abscissa: presynaptic potential change; ordinate: arbitrary units.

Hence, at constant $[Ca]_o$, k varies as the nth root of R, and one may derive the relation between input voltage and 'calcium permeability' k, by taking, e.g. in the case of Fig. 8*B*, the 2·7th root of the corrected postsynaptic response at 2·75 mM-$[Ca]_o$. The resulting curve (Fig. 9) is less steep than the input/output curve, but both require correction for presynaptic distortion (factor (*b*) on p. 798) which would cause their slopes at high input voltages to become steeper than shown here.

The corrected curves in Fig. 8 run approximately parallel up to 11 mM-$[Ca]_o$. It follows, therefore, that even at the normal calcium concentration of 11 mM, k and R go on increasing up to quite high input voltages, much

as indicated by the two curves at 2·75 mM-$[Ca]_o$ in Fig. 9. The point of interest in this analysis is this: using normal calcium concentration, and with input potentials equivalent in size to the normal action potential, one may well reach the flat part of the *uncorrected* input/output curve; but after correcting for pre- and post-synaptic voltage distortions, one finds that the action potential may still operate on a fairly steep slope of the relation between transmitter release and presynaptic potential change.

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REFERENCES

BLOEDEL, J. R., GAGE, P. W., LLINÁS, R. & QUASTEL, D. M. J. (1967). Transmission across the squid giant synapse in the presence of tetrodotoxin. *J. Physiol.* **188**, 52–53*P*.

DODGE, F. A. JR. & RAHAMIMOFF, R. (1967). Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *J. Physiol.* **193**, 419–432.

HUBBARD, J. I., JONES, S. F. & LANDAU, E. M. (1968). On the mechanism by which calcium and magnesium affect the release of transmitter by nerve impulses. *J. Physiol.* **196**, 75–86.

JENKINSON, D. H. (1957). The nature of the antagonism between calcium and magnesium ions at the neuromuscular junction. *J. Physiol.* **138**, 434–444.

KATZ, B. & MILEDI, R. (1967). A study of synaptic transmission in the absence of nerve impulses. *J. Physiol.* **192**, 407–436.

KATZ, B. & MILEDI, R. (1969*a*). Tetrodotoxin-resistant electric activity in presynaptic terminals. *J. Physiol.* **203**, 459–487.

KATZ, B. & MILEDI, R. (1969*b*). The effect of divalent cations on transmission in the squid giant synapse. *Pubbl. Staz. zool. Napoli* **37**, 303–310.

MARTIN, A. R. (1955). A further study of the statistical composition of the end-plate potential. *J. Physiol.* **130**, 114–122.

MILEDI, R. (1967). Spontaneous synaptic potentials and quantal release of transmitter in the stellate ganglion of the squid. *J. Physiol.* **192**, 379–406.

MILEDI, R. (1969). Transmitter action in the giant synapse of the squid. *Nature, Lond.* **223**, 1284–1286.

MILEDI, R. & SLATER, C. R. (1966). The action of calcium on neuronal synapses in the squid. *J. Physiol.* **184**, 473–498.

TAKEUCHI, N. (1963). Effects of calcium on the conductance change of the end-plate membrane during the action of transmitter. *J. Physiol.* **167**, 141–155.

YOUNG, J. Z. (1939). Fused neurons and synaptic contacts in the giant nerve fibres of cephalopods. *Phil. Trans. R. Soc.* B **229**, 465–503.